Journal of Medical Virology 59:480–490 (1999)

Emergence and Genetic Evolution of HIV-1 Variants With Mutations Conferring Resistance to Multiple Reverse Transcriptase and Protease Inhibitors

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The emergence of genotypic resistance in protease and reverse transcriptase (RT) gene regions was longitudinally evaluated in plasma samples from a group of 12 HIV-1–infected patients treated with different combination of antiretroviral therapies and selected on the basis of their clinical failure. Complex mutational patterns in the reverse transcriptase gene were observed. In particular, combinations of AZT (41L, 67N, 70R, 210W, and 219Q/E) and 3TC (184M) were seen in 10 patients. Two patients presented codon 151 multinucleoside analogue resistance (MNR). Additionally, seven patients harbored RT nonnucleoside analogue-related resistance substitutions (98G, 103N, and 181C). Multiple protease-selected mutations were found in each patient with an average of six substitutions per patient, with 10I/F/V, 63P, 71V, 82A/T, 84V, and 90M being the most prevalent substitutions. Overall, these results showed that for most patients virological failure was coupled with detectable genotypic resistance. Furthermore, most patients exhibited genotypic resistance to almost all available anti–HIV-1 drugs. The high viral loads found in most patients at the end of the study suggest that the replication of these multidrug resistant viruses are not severely compromised. Phylogenetic analysis of these *pol* sequences revealed that a specific HIV-1 genotype prone to develop multidrug resistance was not found. ***J. Med. Virol. 59:480–490, 1999.***

KEY WORDS: reverse transcriptase; HIV-1; protease inhibitor

INTRODUCTION

The use of potent combination antiretroviral therapies [highly active antiretroviral therapy (HAART)] for the treatment of human immunodeficiency virus type 1 (HIV-1)-infected individuals can reduce plasma HIV-1 RNA to levels below the limit of detection for long periods of time [Gulick et al., 1997; Hammer et al., 1997; Arnó et al., 1998]. However, the development of resistance to antiretroviral drugs decreases the benefit of combination therapy [Carpenter et al., 1997; Domingo et al., 1997]. Drug resistance has rarely been reported in drug-naive HIV-1–infected patients treated with HAART [Finzi et al., 1997; Günthard et al., 1997; Wong et al., 1997; Martínez et al., 1999], but prior exposure to antiretroviral drugs strongly increases the risk of the emergence of drug-resistant variants [Collier et al., 1996; D'Aquila et al., 1996]. Although treatment failure is a complex phenomenon [Kaufmann et al., 1998; Perrin and Talenti, 1998], the widespread use of mono or double combination therapies before the introduction of HAART may be an important drawback in the treatment of HIV-1–infected patients. Recently, the presence of multiple reverse transcriptase (RT) and protease mutations was described in four patients that confers high-level resistance to zidovudine, lamivudine, saquinavir, indinavir, and nelfinavir, and low-level resistance to didanosine, zalcitabine, and stavudine [Shafer et al., 1998]. Obviously, the fact that some patients may develop multidrug resistance reduces HIV-1 therapeutic options. The need for the development of therapies against multiple antiretroviral drug-resistant HIV-1 isolates emphasizes the importance of monitoring the prevalence, evolution, and clinical implications of multidrug resistance in larger populations. It has also been recently reported that viruses carrying substitutions that confer multidrug resistance can be transmitted [Hecht et al., 1998]. These data

Grant sponsor: irsiCaixa Foundation; Grant number: FIS 98-0054-03.

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Accepted 7 May 1999

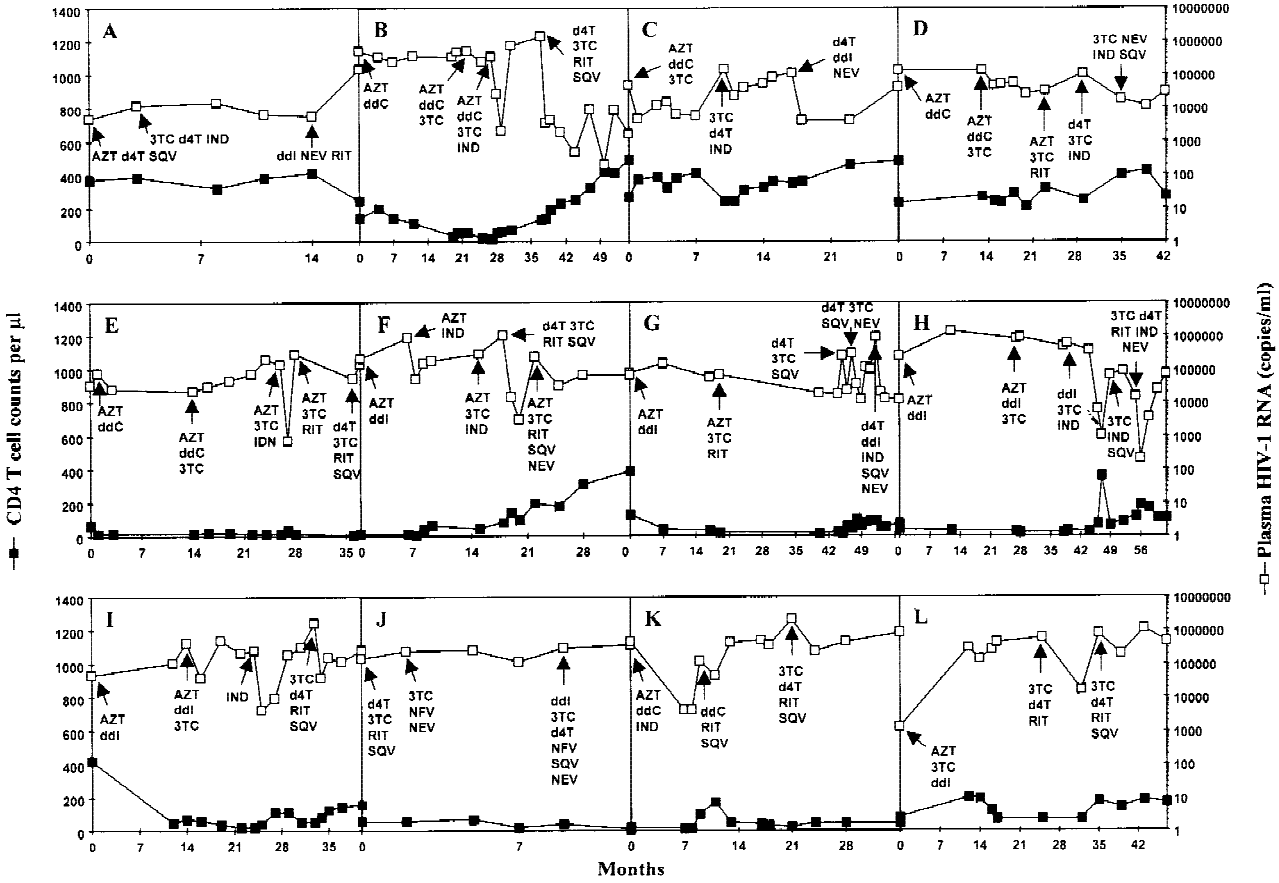

Fig. 1. Summary of CD4 T-cell counts and quantitative HIV-1 virion-associated RNA for each of the 12 infected patients (A through L). The time after the study baseline is annotated on the abscissa. CD4 T-cell counts were measured by flow-activated cytometric assay and expressed per microliter of blood. HIV-1 RNA levels were measured by a quantitative reverse transcription-PCR assay (Roche Molecular Systems) and are expressed per milliliter of plasma. AZT = zidovudine; ddC = zalcitabine; ddI = didanosine; 3TC = lamivudine; d4T = stavudine; NEV = nevirapine; IND = indinavir; SQV = saquinavir; RIT = ritonavir; NFV = nelfinavir.

highlight the increasing problem of HIV-1 multidrug resistance and underline the importance of continued resistance surveillance.

In the present study, the emergence of genotypic resistance in the protease and reverse transcriptase genes was evaluated in plasma samples from a cohort of 12 HIV-1–infected patients that failed clinically different combination antiretroviral therapies. In order to detect genotypic factors implicated in determining multidrug resistance, phylogenetic reconstructions of these HIV-1 *pol* sequences were also performed.

MATERIALS AND METHODS

Patients

Twelve HIV-1–seropositive patients who consecutively failed combination therapies were selected for this study. All these patients had experienced anti–HIV-1 therapy (Fig. 1) that included between 4–5 nucleoside analogs and 1–4 protease inhibitors. Six patients received nevirapine. Table I shows the characteristics of the study cohort during the follow-up period.

HIV-1 RNA Quantification

Plasma virion-associated HIV-1 RNA was measured by a quantitative reverse transcription-PCR assay (Amplicor Monitor assay, Roche Molecular Systems) and is expressed per milliliter of plasma. The detection limit of this assay is 200 copies of HIV-1 RNA per milliliter of plasma.

RNA Preparation, cDNA Synthesis, and PCR

RNA was extracted from a volume of 140 µl of plasma with the QIAamp blood kit (Qiagen). After viral RNA isolation, 10 µl of resuspended RNA (corresponding to 28 µl of plasma) was reverse-transcribed and amplified using Titan one-tube RT-PCR System (Boehringer Manheim) according to the manufacturer's instructions. Briefly, the RT-PCR mixture contained 10 pmol of protease oligonucleotides 5′ prot1 (sense) [5′-AGCTAATTTTTTAGGGAAGATCTGGCCTTCC-3′, HXB2 positions 2077 to 2108 (Myers et al., 1996)] and 3′ prot1 (antisense) (5′-GCACCTACTGGAGTATTG-TATGGATTTTCAGG′-3, HXB2 positions 2703 to 2733), or reverse transcriptase oligonucleotides RT 18

TABLE I. Clinical Characteristics and Treatment Histories of the 12 Study Patients

					At study entry		At study end	
Patient	Age	Sex	Months of follow-up	Disease stage[a]	CD4 T cells/ml	Plasma viral load (copies/ml)	CD4 T cells/ml	Plasma viral load (copies/ml)
A	30	Male	18	B2	194	430196	154	11741
B	36	Male	58	B3	140	525034	489	1659
C	45	Male	28	B2	260	47838	480	41944
D	29	Male	42	B2	220	128455	276	30254
E	28	Female	37	C3	61	32698	10	143405
F	36	Male	34	C3	10	209428	388	66720
G	41	Male	56	C3	128	76823	71	11798
H	34	Female	60	C3	40	252569	110	69322
I	41	Male	40	B2	420	46703	156	252580
J	27	Male	12	C3	54	140000	6	363344
K	48	Male	35	C3	20	444637	46	849285
L	32	Male	47	B3	80	1325	47	468987

[a]CDC [1992].

(sense) (5′-GGAAACCAAAAATGATAGGGGGAATTG-GAGG-3′, HXB2 positions 2376 to 2406) and RT 21 (antisense) (5′-CTGTATTTCTGCTATTAAGTCTTTT-GATGGG-3′, HXB2 positions 3508 to 3538), 200 μM of dNTPs, 1.5-mM $MgCl_2$, RT-PCR buffer, 5-mM DTT, 5 units of RNase inhibitor, and 1 μl of enzyme mix (AMV and Expand High Fidelity PCR System) in a total reaction volume of 50 μl. The samples were incubated for 30 min at 50°C; then 1 cycle of denaturation at 94°C for 2 min; then 10 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 68°C for 1 min; and then 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 68°C for 1 min plus 5 sec for each cycle. A final extension at 68°C for 7 min was added to the last cycle. A 5-μl aliquot was again amplified in a 100-μl reaction mixture containing 10 pmol of protease oligonucleotides 5′ prot 2 (sense) (5′-TCAGAGCAGACCAGAGCCAACAGC-CCCCA-3′, HXB2 positions 2135 to 2162) and 3′ prot 2 (antisense) (5′-GCAAATACTGGAGTATTGTATG-GATTTTCAGG-3′, HXB2 positions 2770 to 2792) or reverse transcriptase oligonucleotides RT 19 (sense) (5′-GGACATAAAGCTATAGGTACA-3′, HXB2 positions 2453 to 2473) and RT 20 (antisense) (5′-CTGCCAGT-TCTAGCTCTGCTTC-3′, HXB2 positions 3440 to 3461), 200 μM of dNTPs, 1.5-mM $MgCl_2$, PCR buffer [50-mM KCl, 10-mM Tris-Cl (pH 8.3)], and 0.5 units of *Taq* DNA polymerase (Perking-Elmer). Cycling parameters were 1 cycle of denaturation at 94°C for 2 min and then 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. This was followed by a 7-min incubation at 72°C. Following the above protocol, the complete protease coding region and the first RT 300 amino acids were amplified.

Sequencing

To verify protease and reverse transcriptase genes amplification and to estimate product yield, 1/20 of the nested PCR mixture was run on a 1.5% agarose gel. PCR products were purified by using the Qiaquick spin PCR purification kit (Qiagen). Sequencing reactions were carried out with the ABI PRISMTM dRhodamine Terminator Cycle Sequencing kit (Applied Biosystems). The products of the reactions were then analyzed on an Applied Biosystems 310 sequencer. Sequencing oligonucleotides for the protease gene were PR2212 (5′-AGCAGGAGCCGATAGACAA-3′, HXB2 positions 2212–2230) and PR2571 (5′-CCTGGCTT-TAATTTTACTGG3′, HXB2 positions 2592–2573), and IN5 (5′-AATTTTCCCATTAGTCCTATTGAAACTG-TACCA-3′, HXB2 positions 2543–2577), Z2447 (5′-GTAGATTTCAGAGAACTTAATA-3′, HXB2 positions 2771–2792), and NE135 (5′-CCCACTAATTCTGTAT-GTCATTGACAGTCCAGCT-3′, HXB2 positions 3333–3299) for the reverse transcriptase gene. Sequence editing was performed using the Sequence Navigator program (Applied Biosystems).

Phylogenetic Analysis

Phylogenetic reconstructions were generated by using the neighbor-joining method in the Phylogeny Inference Package (PHYLIP) [Felsenstein, 1988, 1995] with a Kimura two-parameter distance matrix (programs DNADIST and NEIGHBOR). Nonsynonymous phylogenetic reconstruction was generated with a Kimura formula (program PROTDIST). Bootstrap resampling [Felsenstein, 1985] was applied to the neighbor-joining trees (programs SEQBOOT and CONSENSE) to assign approximate confidence limits to individual branches. The final graphical output was created with the program TREEVIEW [Page, 1996]. The proportion of synonymous substitutions per potential synonymous sites and the proportion of nonsynonymous substitutions per potential nonsynonymous sites were calculated with the program WET [Dopazo, 1995]

Statistical Analysis

The distribution of RNA HIV-1 loads, CD4 T-cell counts, and genetic distances between baseline and end time points from each patient were subjected to nonparametric statistical treatment by using the Wilcoxon signed rank test included in the SPSS version 7.5 soft-

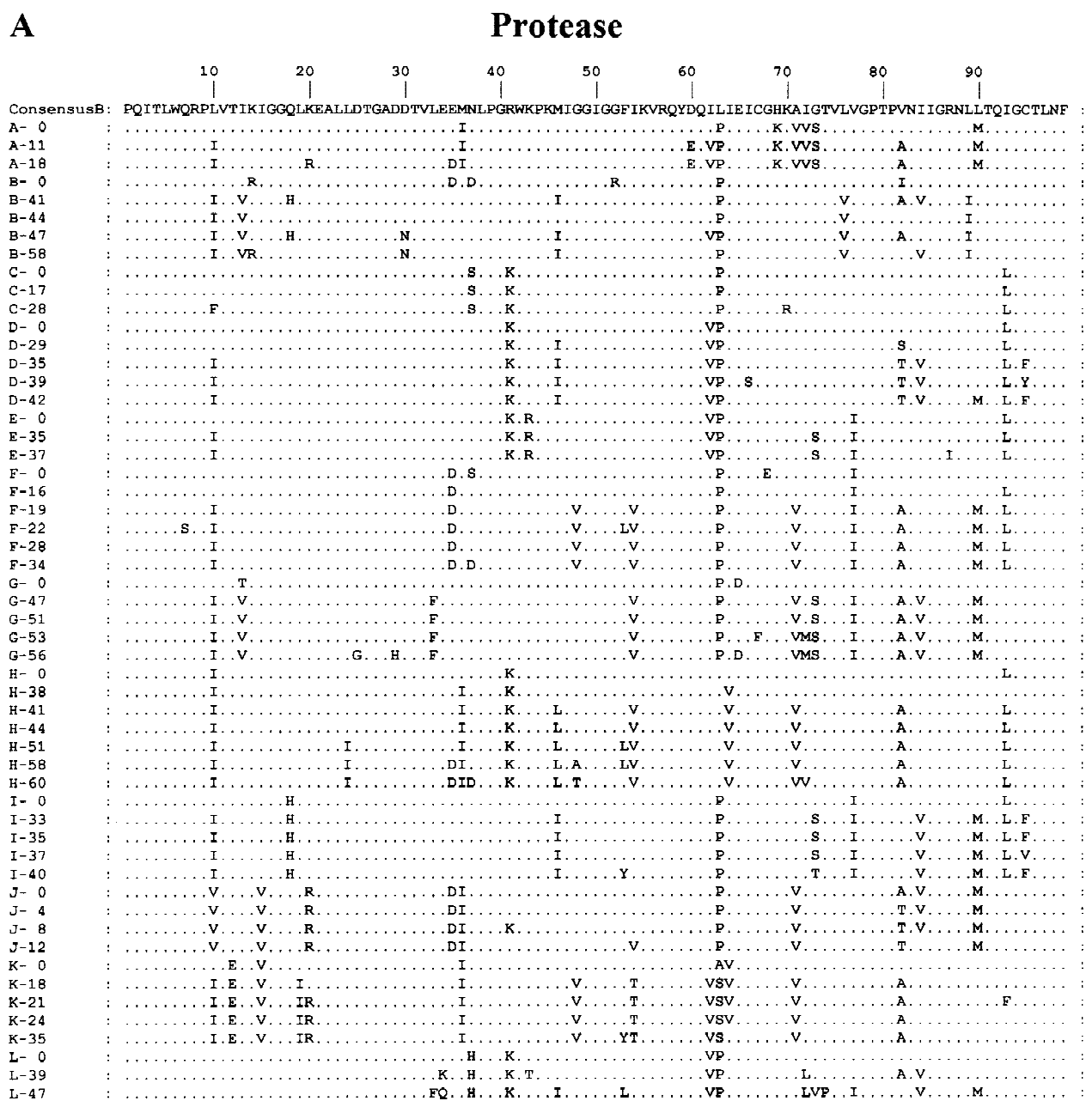

Fig. 2. Deduced amino acid sequence alignments of the **(A)** protease sequences and **(B)** (on next page) reverse transcriptase sequences. The deduced amino acid sequences are annotated by a capital letter for each patient (A through L). For each patient the sample time points are indicated. Amino acid changes relative to the clade B consensus sequence [Myers et al., 1996] are indicated. Dots indicate amino acid sequence identity.

ware package (SPSS, Chicago, IL). Means were calculated as geometric means.

Nucleotide Sequence Accession Numbers

The HIV-1 sequences described in this study have been submitted to GenBank under accession numbers AF102277 through AF102368.

RESULTS

Virological and Clinical Characterization of Study Cohort

CD4 T-cell count and HIV-1 RNA plasma levels were measured in all the patients in the study 12 to 60 months (mean 39 months) of follow-up period. CD4 T-cell count ranged at study entry from 10 CD4 cells/μl to 420 CD4, and at the end of the study ranged from 6 CD4 cells/μl to 489 CD4 (Table I). Six of the 12 patients had an increase in their CD4 T-cell count in response to therapy (Fig. 1), but when the distribution of these values was determined, there was not a significant difference between the two time points ($P = 0.308$, Wilcoxon signed rank test). Plasma viral RNA was also quantified from the initial and final time points (Table I), and the distribution of these values were determined. Again, no statistically significant difference was found

B **Reverse Transcriptase**

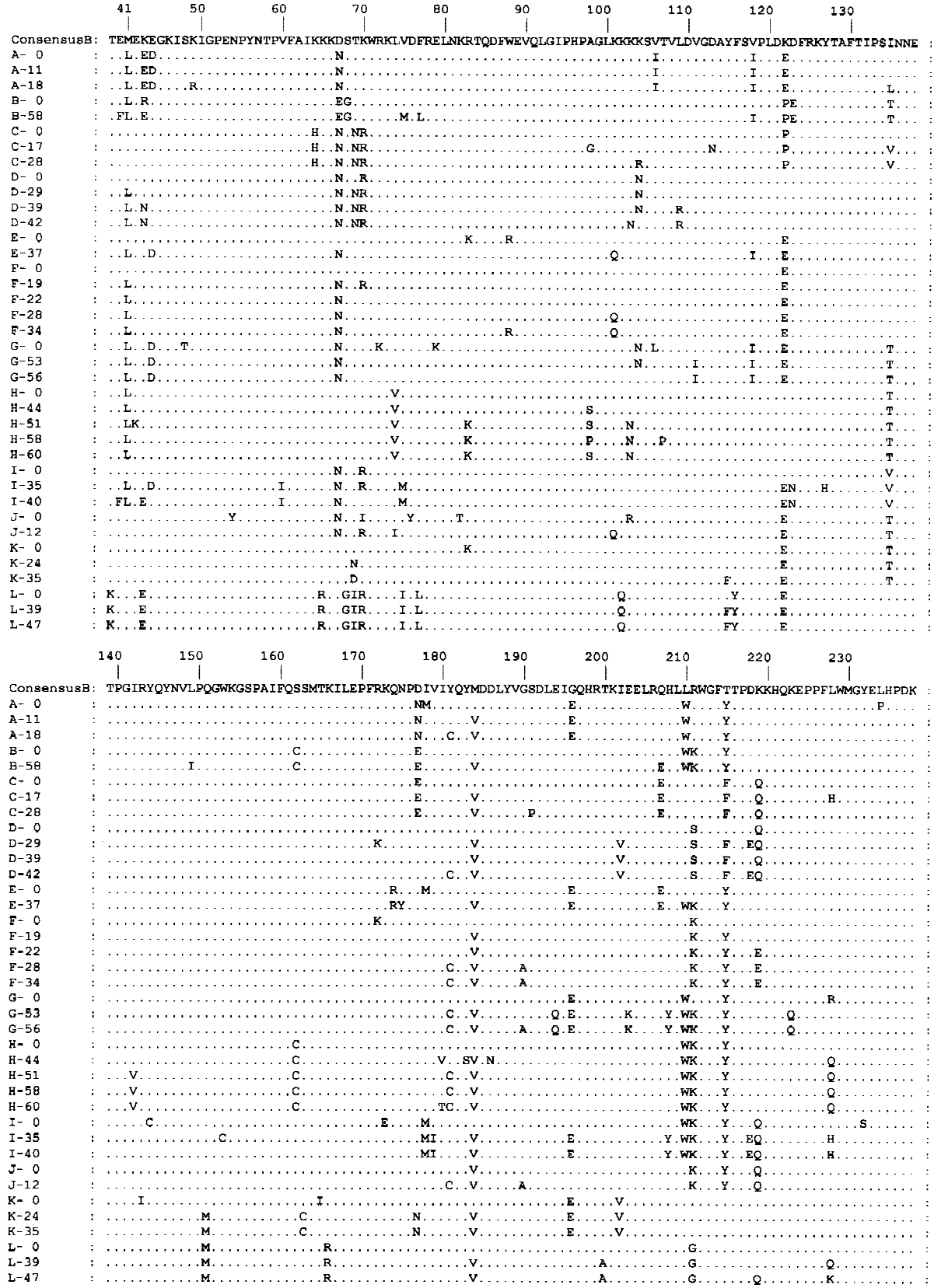

Fig. 2. Continued.

($P = 0.875$). Most patients had a decrease relative to the baseline in HIV-1 RNA levels within 1 month of the beginning of therapy; however, the viremia levels rose as therapy followed. Successive HIV-1 RNA ups and downs were observed, represented in Figure 1, when new therapies were implemented in those patients. These data suggest that treatment success in this study cohort was not achieved after 12 to 60 months of antiretroviral therapy.

Reverse Transcriptase and Protease Sequences

To investigate the mechanisms of therapeutic failure in these 12 patients, the reverse transcriptase and protease genotypic patterns of resistance were analyzed in serial plasma samples derived from each patient. The protease and 720 bp of the reverse transcriptase regions (codon positions 1 to 240) were analyzed by direct cycle sequencing of DNA, which was amplified from reverse-transcribed RNA (Fig. 2A and 2B, respectively). The zidovudine-associated resistance mutations M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E were observed in 8 (66%), 8 (66%), 6 (50%), 6 (50%), 10 (83%), and 6 (50%) of the patients, respectively. The lamivudine (M184V)-associated resistance mutation was found in all the patients. Two patients presented the multidideoxy-nucleoside resistance (MDR) pattern; patient L showed a set of four mutations (V75I, F77L, F116Y, and Q151M) and patient K two mutations (F116Y and Q151M). Stavudine-, didanosine-, and zalcitabine-associated resistance substitutions were rarely seen although all the patients had experience with at least one of these nucleoside analogues. Only two patients, H (L74V), and K (T69D), had substitutions related with the former drugs. Of note is that substitution T69D found in patient K coexisted with the MDR substitutions F116Y and Q151M. This resistance pattern is described here for the first time [Schmit et al., 1996; Kavlick et al., 1998]. The AZT-associated resistance substitution K70R rarely coexisted with the substitution L210W; interestingly, within patient I these two coexisted for a while at early time points but the substitution K70R was not detected in the final time point (Fig. 2B). The recently described 6-bp insertion between codons 68 and 70 that arise after different nucleoside analogues treatments [Jong et al., 1998; Whitcomb et al., 1998; Winters et al., 1998] was not found in any patient in the present study cohort. The six patients treated with the nonnucleoside reverse transcriptase inhibitor nevirapine had nevirapine-associated resistance substitutions. Thus, the nonnucleoside-associated resistance substitutions K103N, V106A/I, Y181C, and G190A were found in 2 (16%), 1 (8%), 6 (50%), and 3 (25%) patients, respectively. In one patient (C), the nevirapine-associated resistance substitution A98G was present without previous exposure to this drug. Finally, reversion of drug resistance substitutions after cessation of different drugs, especially AZT, were rarely seen. Only the above-mentioned K70R substitution present in patient I was found to revert while the L210W substitution developed (Fig. 2B).

Multiple protease-selected substitutions were found in each patient (Fig. 2A). An average of six protease inhibitor-associated substitutions were present at endpoint, with L10I/F/V (92%), L63P (83%), V82A/T (83%), I84V (50%), A71V (50%), and L90M (50%) being the most prevalent substitutions. As mentioned for the reverse transcriptase-associated resistance substitutions, reversion of drug resistance substitutions after cessation of different protease inhibitors were not observed (Fig. 2A). As expected, sequential samples showed the accumulation of mutations over time. No correlation between the type and number of substitutions was found in the multidrug-resistant proteases and a particular reverse transcriptase genotype, since this correlation was more easily found with the number of protease inhibitors received and time on therapy. For instance, patient C who had only two protease inhibitor resistance substitutions is the only patient in the present study cohort that received just one protease inhibitor during a short period of time (Figs. 1 and 2A).

These results showed that in the majority of patients the virological failure was coupled to detectable genotypic resistance. Moreover, most patients exhibited genotypic resistance to almost all available anti–HIV-1 drugs. The high viral loads observed in the present study cohort at endpoint, not significantly different from the initial values, also suggest that the replication efficiency of these multidrug-resistant viruses is not severely compromised.

Phylogenetic Analysis of the Protease and Reverse Transcriptase Regions

To analyze the evolutionary trends of multidrug-resistant HIV-1 isolates, DNA sequence distances were computed from a pairwise comparison of protease and reverse transcriptase sequences sequentially obtained from each patient. Protease and reverse transcriptase neighbor-joining DNA trees constructed by using these distances values are shown in Figure 3. Distinct clustering of sequences corresponding to each patient were found, indicating absence of PCR product cross-contamination. Viral sequence changes were observed at each time point, indicating continuous viral evolution in all individuals. The rate of viral evolution was derived for each patient by analysis of the protease and reverse transcriptase distances extracted from the intrapatient point DNA distance values used to construct the neighbor-joining trees. For each patient, the rate of HIV-1 evolution was tracked by calculating the genetic distances per nucleotide and per months on therapy (Table II). When the two *pol* regions were analyzed, protease sequences showed a higher rate of evolution per month on therapy (mean $= 1.7 \times 10^{-3} \pm 05 \times 10^{-3}$ sub/nt/month) compared with the reverse transcriptase sequences (mean $= 0.8 \times 10^{-3} \pm 0.4 \times 10^{-3}$ sub/nt/month), and this difference was statistically significant ($P = 0.006$, Wilcoxon signed rank test).

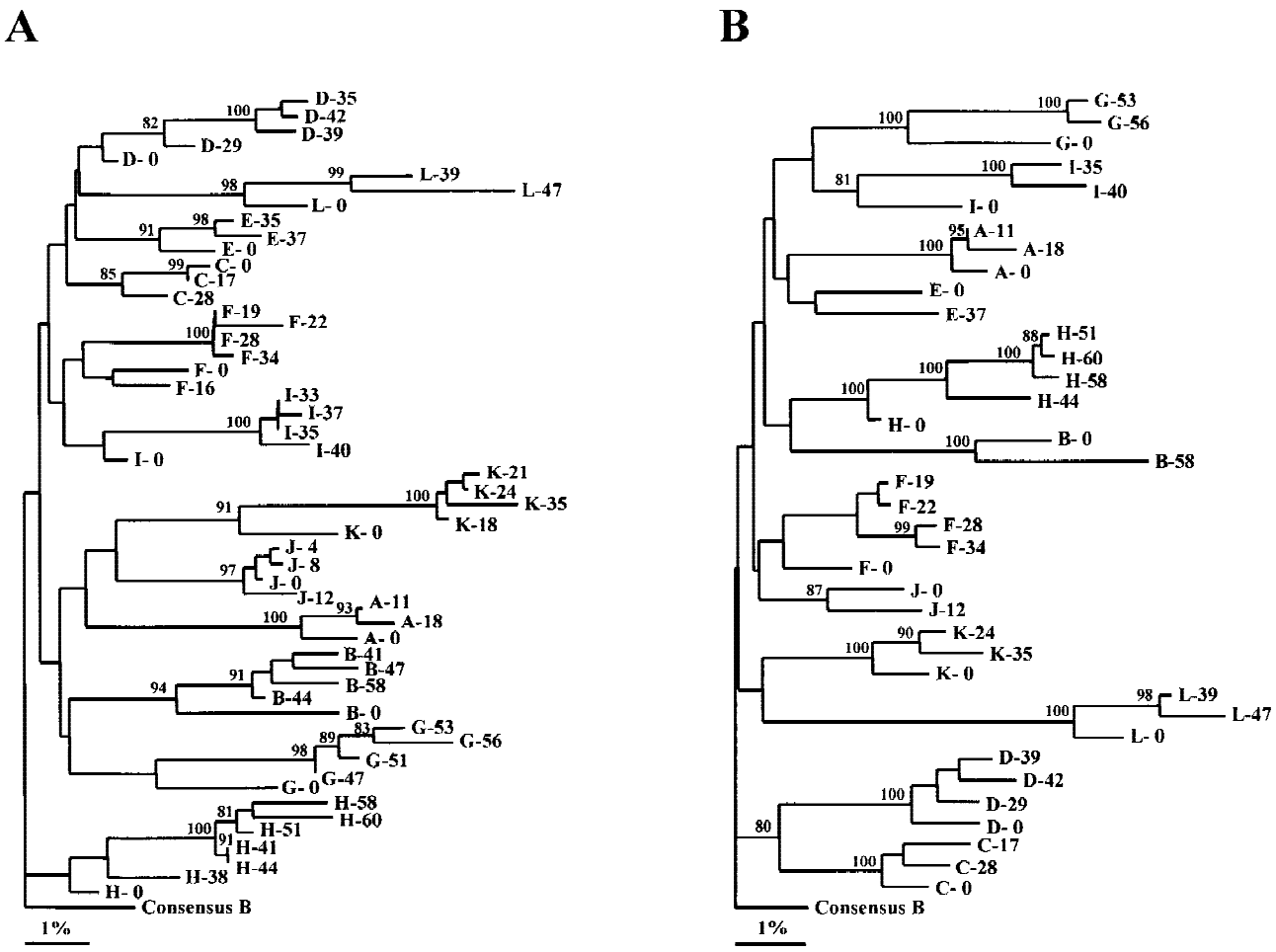

Fig. 3. Neighbor-joining phylogenetic tree of viral nucleotide sequences from the plasma of the 12 patients analyzed in this study. **A:** Protease sequences. **B:** Reverse transcriptase sequences. The patients are annotated by a capital (A through L). For each patient the sample time points are indicated. Numbers at branch nodes refer to the number of bootstrap repetitions (of 100) at which the distal sequences grouped together; only those occurring at a frequency greater than 80% are shown. Clade B consensus sequence [Myers et al., 1996] was used as an out group.

A similar calculation was performed by comparing the distribution of interpatient DNA point distances for the initial and final time points (Table III). A statistically significant increase in sequence diversity was observed in the two regions analyzed (protease and reverse transcriptase) for the sequences obtained at the final time point ($P < 0.001$ and $P < 0.001$, respectively; Table III). As expected, this increase in sequence diversity was due to a statistically significant increase in the number of nonsynonymous substitutions ($P < 0.001$ and $P < 0.001$; Table II). The initial synonymous sequence diversity was not significantly different from the final time point ($P = 0.934$ for the protease gene; Table III). Because at study entry 10 out of 12 patients had nucleoside analogue experience, a more modest increase in the number of nonsynonymous substitutions was observed in the reverse transcriptase gene (Table III). Thus, the amount of nonsynonymous substitutions rose during the time on therapy but the number of silent substitutions remained static.

Finally, to assess whether a certain genetic background may predispose HIV-1 to develop multidrug resistance, phylogenetic analysis based on only nonsynonymous changes of *pol* sequences obtained at baseline was carried out. In this analysis, only protease sequences were analyzed because at baseline 10 out of 12 patients had nucleoside analogue experience. In contrast, only two patients, A and J, had received protease inhibitors at baseline. Protease sequences from the HIV-1 data bank were included in the analysis in order to search for a specific amino acid background prone to develop protease multidrug resistance. The results of the protease phylogenetic analysis by the neighbor-joining method are shown in Figure 4. The gene analyzed showed a phylogenetic tree without consistent topological patterns or clusters; that is, low bootstrap values representing different sequence groups were found. Different HIV-1 data bank sequences clustered with different sequences from this study. Furthermore, clusters of sequences from the present study were not found, suggesting the absence of a common genetic background in this cohort. Overall, these results did

TABLE II. Intrapatient Protease and Reverse Transcriptase Genetic Distances

Genomic region	Patient	Months on therapy	Substitutions	Rate of evolution (s/nt/month)[a]	Non (%)[b]	Syn (%)[b]
Protease	A	18[c]	7	1.3×10^{-3}	86	14
	B	30	13	1.4×10^{-3}	85	15
	C	19	6	1.1×10^{-3}	33	67
	D	22	11	1.7×10^{-3}	55	45
	E	10	7	2.3×10^{-3}	43	57
	F	19	13	2.3×10^{-3}	85	15
	G	36	14	1.3×10^{-3}	93	7
	H	23	15	2.2×10^{-3}	73	27
	I	16	10	2.1×10^{-3}	70	30
	J	10	4	1.3×10^{-3}	75	25
	K	35	18	1.7×10^{-3}	61	39
	L	22	15	2.3×10^{-3}	67	33
	Mean ± SD	21.6	11.1	1.7×10^{-3} 0.5×10^{-3}	69	31
Reverse transcriptase	A	18[d]	9	0.8×10^{-3}	67	33
	B	58	21	0.6×10^{-3}	38	62
	C	28	11	0.6×10^{-3}	36	64
	D	42	19	0.7×10^{-3}	58	42
	E	37	20	0.8×10^{-3}	63	37
	F	34	22	1.0×10^{-3}	55	45
	G	56	30	0.8×10^{-3}	57	43
	H	60	17	0.4×10^{-3}	47	53
	I	40	28	1.1×10^{-3}	61	39
	J	12	15	1.9×10^{-3}	60	40
	K	35	15	0.7×10^{-3}	67	33
	L	47	16	0.5×10^{-3}	38	62
	Mean ± SD	38.9	18.6	0.8×10^{-3} 0.4×10^{-3}	54	46

[a]Substitutions per nucleotide per month.
[b]Non and syn correspond to the percentage of nonsynnonymous and synnonymous substitutions relative to the first subject sample sequence.
[c]Months on protease inhibitors therapy.
[d]Months on reverse transcriptase inhibitors therapy.

not allow the identification of a specific genotype or amino acid background that might predispose to the present HIV-1 isolates to develop multidrug resistance in the protease gene.

DISCUSSION

We have characterized fragments of the viral *pol* gene sequences for variation over time in 12 HIV-1–infected patients who failed consecutively different drug combination regimens. The results show that HIV-1 can acquire, independently of its genetic background, resistance to all available anti–HIV-1 drugs. Of note is that the accumulation of a high number of substitution in one gene did not hamper the development of resistance to new drugs directed at the same target. For instance, the development of nonnucleoside analogues drug resistance (Fig. 2B) was not affected by the presence of different patterns of nucleoside analogue resistance. As expected, neither affects the development of resistance in a different gene. The neighbor-joining algorithm revealed clusters in protease sequences that generally were not present in the reverse transcriptase phylogenetic reconstruction, suggesting that the protease resistance pattern observed above was not affected by the different reverse transcriptase sequences (Fig. 3). In agreement with the *pol* genetic plasticity shown here, there is the recently described reverse transcriptase insertion at position 69, which was also detected in heavily treated patients failing combination therapy [Jong et al., 1998; Whitcomb et al., 1998; Winters et al., 1998].

The data presented above are in agreement with a recent study that found four HIV-1 isolates that shared seven protease mutations and eight reverse transcriptase mutations that showed high-level resistance to zidovudine, lamivudine, saquinavir, indinavir, and nelfinavir and lower level of resistance to didanosine, zalcitabine, and stavudine [Shafer et al., 1998]. We found sequences with a higher number of substitutions (Fig. 2), probably due to the fact that the patients studied here were analyzed later and therefore have received more drugs and over a longer period of time. This is particularly true for the protease gene where after a long period of therapy with different protease inhibitors similar patterns of resistance, which included the mutations 48V, 82A, and 90M (Fig. 2A), was reached. Viruses with these mutations have been recently shown to be highly resistant to ritonavir, indinavir, and saquinavir [Hertogs et al., 1998]. From a clinical point of view, patients at the advanced stage of disease harboring viruses with multiple protease inhibitor resistance substitutions should not receive protease inhibitors because mainly toxicity rather than efficacy can be expected. We have identified a more heteroge-

TABLE III. Intertime Point Distances for Protease and Reverse Transcriptase Sequences

Coding region	DNA distance (mean ± SD)[a]			Syn[b] (mean ± SD)			Non[c] (mean ± SD)		
	At study entry	At study end	*P* value[c]	At study entry	At study end	*P* value[c]	At study entry	At study end	*P* value[c]
Protease	0.057 ± 0.017	0.089 ± 0.021	<0.001	0.118 ± 0.044	0.122 ± 0.044	0.934	0.035 ± 0.013	0.071 ± 0.015	<0.001
Reverse transcriptase	0.059 ± 0.014	0.077 ± 0.017	<0.001	0.140 ± 0.037	0.169 ± 0.032	<0.001	0.034 ± 0.003	0.046 ± 0.014	<0.001

[a]DNA distance corresponds to the mean proportion of substituted nucleotides when pairs of different patients sequences were compared by applying the Kimura two-parameter model.
[b]Syn and non corresponds to the mean proportion of synonymous and nonsynonymous substituted nucleotides when pairs of different patients sequences were compared.
[c]Wilcoxon signed rank test.

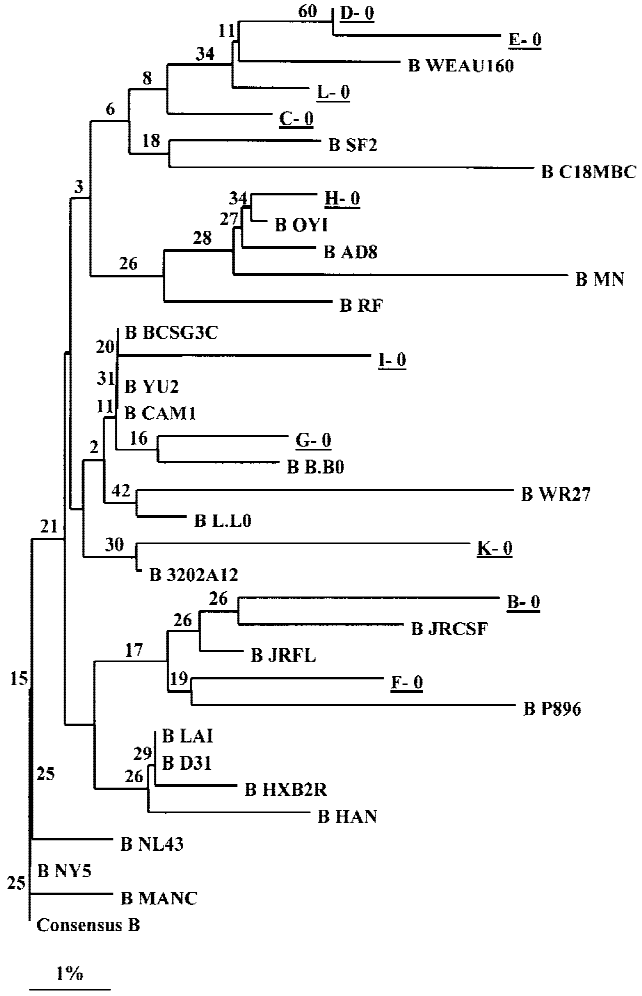

Fig. 4. Neighbor-joining phylogenetic tree of viral protease sequences based in nonsynonymous substitutions from the plasma of 10 patients analyzed in this study. Only sequences from the study baseline were included in this phylogenetic analysis. Patients A and J were not included in the analysis because at baseline they were not naive for protease inhibitors. Study patients are annotated as capital letters and are underlined to facilitate their identification. The following subtype B protease sequences were taken from the HIV data bank [Myers et al., 1996] and used in this phylogenetic reconstruction: B WEAU160, B SF2, B C18MBC, B OYI, B AD8, B MN, B RF, B BCSG3C, B YU2, B CAM1, B B.B0, B WR27, B 3202A12, B JRCSF, BJRFL, B P896, B LAI, B D31, B HXB2R, B HAN, B NL43, B NY5, and B MANC. Numbers at branch nodes refer to the number of bootstrap repetitions (of 100) at which the distal sequences grouped together. Clade B consensus amino acid protease sequence [Myers et al., 1996] was used as an out group.

neous pattern of multidrug resistance, especially in the reverse transcriptase gene. As mentioned above, a larger number of substitutions and different and more complex pattern of multidrug resistance will be found when more HIV-1 isolates from heavily treated patients are analyzed in the future. Interestingly, as shown by the phylogenetic analysis of Figure 3, viruses with a different genetic background are able to develop the same pattern of drug resistance (Fig. 2), suggesting a lack of genetic restriction in the development of drug resistance. In agreement with this finding is that treatment with zidovudine and lamivudine (in different

regimens) can select for the same RT substitutions, T215Y and M184V, respectively, in group O viruses as those found in zidovudine and lamivudine-resistant group M viruses [Quiñones-Mateu et al., 1998].

An important aspect of the present study is that no apparent common genetic background was identified that might predispose to the present HIV-1 isolates to develop multidrug resistance in the protease gene. As shown by the low bootstrap values observed in the phylogenetic analysis presented here, where sequences from the HIV-1 data bank [Myers et al., 1996] were included (Fig. 4), a specific genotype or amino acid background more predisposed to develop protease multidrug resistance was not found. Recently, a common genetic background genotype that forced the development of the different multidrug resistance patterns observed in the reverse transcriptase gene was not detected [Kavlick et al., 1998]. The small viral effective population size model, in which chance plays a significant role [Leigh-Brown, 1997 and Leigh-Brown and Richman, 1997], has been suggested as an explanation for different resistance patterns found in similar patients. Previous studies have shown that HIV-1 viral background plays a major role in development of resistance to protease inhibitors [Rose et al., 1997] and that some single substitutions that confer resistance to different antiretroviral drugs directed to reverse transcriptase and protease genes can impair the HIV-1 replicative capacity [Larder et al., 1995; Markowitz et al., 1995; Back et al., 1996; Borman et al., 1996; Harrigan et al., 1996; Croteau et al. 1997; Sharma and Crumpacker, 1997]. Estimation of the effects of drug resistance on viral replication capability is important since it has been postulated that less-fit viruses might result in clinical benefits [Coffin, 1995], as recently has been shown by Black et al. [1998] who found that a proportion of long-term survivors had less-fit NSI viruses. The high viral loads of most patients of this study after 12 to 60 months of follow-up not far away from those present at baseline demonstrated the apparent ease for the drug-resistant viruses to develop compensatory substitutions to improve their replicative capacity. Many protease-associated drug resistance substitutions found above and elsewhere do not confer drug resistance, but are compensatory substitutions that increase the HIV-1 replication capacity [Nijuis et al., 1997; Matínez-Picado et al., 1999].

Taken together, these data showed that drug-associated resistance substitutions to all available drugs may occur. Additionally, no HIV-1 genetic background was identified that limited the capacity for viral evolution and drug resistance development. As suggested before, these data highlight the dangers of selecting suboptimal sequential antiretroviral therapies for HIV-1–infected persons [Condra, 1998]. Although the cost of resistance testing is a concern, it seems that the cost of the assays is small when contrasted with the cost of prescribing an ineffective antiretroviral regimen. In addition, sparing patients unnecessary adverse effects may be possible if resistance testing identifies which drugs are unlikely to work. The increasing number of heavily drug-experienced patients receiving multiple drugs regimenes warrants a large-scale study of the multidrug resistance prevalence and its clinical implications.

ACKNOWLEDGMENTS

M.C. was supported by a predoctoral fellowship from irsiCaixa Foundation.

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